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PRINCIPAL INVESTIGATOR: Anita Lal, Ph.D.

CONTRACTING ORGANIZATION: Regents of the University of California
San Francisco CA 94143-0962

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14. ABSTRACT This proposal is concerned with the functional role of merlin in arachnoidal and meningioma cells. The focus of year 1 was to characterize and develop meningioma-specific NF2 model systems. We have successfully generated three additional meningioma cell lines. The expression of merlin was quantitated at the transcript and protein level in these and arachnoidal cells. The KT21MG1 cell line is merlin-deficient and contains a novel splice site mutation between exon 14 and 15. This alternative splicing results in a 79 bp insertion that encodes for truncated merlin. We have used a tetracycline inducible system, to express wild type merlin and the L64P, S518A and S518D mutant forms of merlin in KT21MG1. Additionally, we have tested the ability of three NF2-specific short interfering RNA (siRNA) to silence merlin. Two siRNAs significantly reduced transcript and protein levels of merlin in arachnoidal cells and the SF6717 meningioma cell line. Stable suppression of merlin in these cell lines has been achieved using the pSUPER retroviral RNAi system. In summary, we have successfully developed three meningioma-specific NF2 model systems. In the subsequent 2 years, we will use these systems to understand the mechanistic role of merlin and to investigate meningioma cell type specific functions.					
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INTRODUCTION

Neurofibromatosis 2 (NF2) is a cancer predisposition syndrome that is phenotypically characterized by the presence of multiple benign brain tumors, primarily schwannomas and meningiomas. Intracranial meningiomas, often multicentric, occur in ~50% of NF2 patients and are associated with an increased risk of mortality. The majority of studies evaluating the mechanism of action of the NF2 gene product, merlin, have used cell lines unrelated to NF2 target cells. Since tumor suppressor function is cell-type specific, the relevance of these studies to the function of merlin in meningiomas is unclear. Additionally, conditional NF2 mouse modeling studies and genotype-phenotype correlation studies suggest mechanistic differences in the function of NF2 between schwannomas and meningiomas. The purpose of this study is to develop meningioma-specific NF2 model systems and to use these model systems to investigate the specific tumor suppressive function of NF2 in meningioma development. Our strategy is to isolate and immortalize human meningioma and arachnoidal cell lines, characterize the expression of merlin in these *in vitro* systems and to engineer merlin-deficient or merlin-expressing meningioma and arachnoidal cells in the relevant genetic backgrounds. These NF2 model systems will be used to dissect the pathways and mechanisms by which merlin expresses its growth suppressive effects specifically in meningioma tumorigenesis.

BODY

Outlined below is a summary of the research accomplishments associated with each task outlined for year 1 in the approved Statement of Work. Tables and Figures related to the text are included in the Supporting Data Section. Appendix 1 includes a recent publication from my laboratory and we specifically mention Appendix 1 when referring to a figure in this manuscript. For example, (Figure 1 in Appendix1) would imply Figure 1 in the attached manuscript in Appendix 1 while (Figure 1) refers to the Supporting Data Section.

Task 1. Characterize merlin expression in immortalized meningioma cell lines (Months 1- 24).

- a. Three additional immortalized meningioma cell lines will be generated using telomerase alone or in conjunction with the human papillomavirus E6/E7 oncogene (Months 1-6).

Meningioma cell lines are very resilient to growth in culture, undergoing irreversible growth arrest after a few passages in culture. In our preliminary data, we had shown that the senescence of meningioma cell lines can be overcome by immortalization with telomerase alone or in conjunction with the human papillomavirus E6/E7 oncogenes. We have recently published this data and the manuscript is included as Appendix 1 (1). This manuscript focuses on three meningioma cell lines: SF3061, SF4068 and SF4433. Immortalized meningioma cell lines maintain morphological, immunocytochemical and genetic features characteristic of the primary meningioma tumor from which they were derived (Figure 2,3,4,6 and 7 in Appendix 1). Using a similar strategy, we have successfully isolated and immortalized three additional meningioma cell lines designated SF6717, SF6747 and SF6561. In addition, we have isolated arachnoidal cells from three independent surgical resections designated SA1, SA2 and CA1. SA1 and SA2 were derived from the spinal meninges while CA1 was derived from the cranial meninges. These arachnoidal cells were immortalized in a manner similar to the meningioma cell lines. Thus, we currently have a total of nine meningioma and three arachnoidal cell lines derived from all three histopathological grades of meningiomas in my laboratory (Table 1). They are representative of a diverse spectrum of meningiomas.

- b. Merlin-deficient cell lines will be chosen using western blot analysis (Months 4-7).

To assess merlin expression in meningioma and arachnoidal cells, we performed western blot analysis on total cell lysates using the C18 polyclonal antibody (Santa Cruz Biotechnology), which is specific for the C-terminal of merlin. This antibody gave us a disproportionate number of non-specific bands around 68 kDa (data not shown) such that it was difficult to decipher merlin expression. We, therefore, tested the A19 polyclonal antibody (Santa Cruz Biotechnology) by western analysis (Figure 1). The A19 antibody is specific for the N-

terminal of merlin and other researchers have found that it often does not react with truncated merlin because these truncated proteins are rapidly degraded (2). A clear protein product of 68 kDa corresponding to isoform 1 of merlin was observed (arrow in Figure 1). The A19 antibody reacted with additional higher molecular weight bands that were deemed to be non-specific since this reactivity was also present in a control human mesothelioma cell line (H-Meso) that is known to be negative for merlin. All the meningioma cell lines tested except KT21MG1 were positive for merlin (Figure 1). This was unexpected given that over 50% of primary meningioma tumors usually have merlin loss and suggested that growth in cell culture selected for cells that were merlin positive. It also showed that our initial plan of expressing merlin in merlin-deficient meningioma cell lines was only applicable to KT21MG1 and that we would have to use siRNA to silence merlin expression in merlin-positive meningioma cell lines using the strategy outlined in Specific Aim 2.

Because of the multiple bands observed by western blot analysis, we felt it necessary to confirm merlin status of the meningioma cell lines using quantitative PCR and immunofluorescence. Immunofluorescence has the added advantage of assessing the correct intracellular localization of merlin. By immunofluorescence, once again, all meningioma cell lines were positive for merlin except KT21MG1 (Figure 2; and Figure 5 in Appendix 1). The meningioma cell lines were co-stained with phalloidin. Phalloidin binds to F-actin localized on membranous ruffles and this co-staining showed that merlin was correctly localized to membrane ruffles in merlin-positive meningioma cell lines (3). The PCR conditions and primers used for quantitative PCR of NF2 are described in the Materials and Methods section of Baia et al (Appendix 1). Once again, all meningioma cell lines, except KT21MG1, were positive for NF2 (Figure 3A). Interestingly, KT21MG1 had a PCR amplicon that was larger than the predicted 234 bp (Figure 3B), suggesting that NF2 had an insertion mutation in KT21MG1. We therefore sequenced the PCR amplicon from KT21MG1. The PCR primers used hybridize to sequences on Exon 14 and Exon 15 and KT21MG1 had a 79 bp insertion that matched the sequence of the intronic region between exon 14 and exon 15 (Figure 4). The first G of the intron was substituted with an A, suggesting that it was a cryptic splice site mutation that resulted in partial intron retention and the consequent insertion of the 79 bp. This insertion leads to a truncated protein at the site of the splicing mutation. A G→A transition in intron 5 resulting in aberrant splicing has been reported earlier for NF2 (4). However, this is the first report of a G→A transition at the first position of intron 15 (5). In fact, mutations in this region of the NF2 gene are considered rare. We looked for a similar mutation in other meningioma tumors. We screened 40 primary sporadic meningiomas using the primer pair described above but did not find this mutation (Figure 5). Thus, we unexpectedly discovered a novel splice site mutation for NF2. It is known that KT21MG1 has loss of one NF2 allele (6) and we have shown that the second allele has a novel splice site mutation resulting in complete inactivation of the NF2 gene.

c. Vector-control and merlin-expressing stable clones will be generated in appropriate meningioma cell lines (Months 6-12).

Our plan was to express merlin isoform 1 in merlin deficient meningioma cell lines using the pLNCX2 retroviral vector. Since merlin is a tumor suppressor and expression of merlin would reduce cell proliferation, we reasoned that a tetracycline-inducible promoter system, where we could control the onset and the levels of expression of merlin, would be a great advantage. We initiated a collaboration with Dr. David Gutmann (Washington University School of Medicine) and received constructs for wild-type NF2 and three specific mutants of NF2 (L64P, S518A and S518D) in the pUHD10-3 plasmid (7, 8). Other components for generating this model system were part of the Tet-On Advanced Inducible Gene Expression System (Clontech). In this system, transgene (in our case, merlin) expression is under the control of a promoter that contains the tetracycline-response element (TRE). The reverse tetracycline transactivator (rtTA) binds to the TRE and activates gene transcription in the presence of doxycycline, a tetracycline derivative. Thus, generating this model system involves insertion of rtTA followed by insertion of merlin into the cell line of interest.

We transfected KT21MG1 with the rtTA vector and selected for stable clones using 600 µg/ml G418. These clones were screened for expression of rtTA using a firefly-luciferase reporter under the control of the

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TRE-promoter. Firefly-luciferase activity was normalized for transfection efficiencies by cotransfecting with Renilla luciferase. Firefly-luciferase activity in the presence of 1 µg/ml doxycycline was increased in individual clones from 113 to 1163-fold (Figure 6). KT21MG1 clone 16 had the highest levels of luciferase activity in the presence of doxycycline and was therefore, selected for further transfections. Wild-type merlin and the L64P, S518A, S518D mutants were transfected into KT21MG1 clone 16 and stable clones were selected with 250 µg/ml hygromycin B. Thus, we have generated stable clones expressing merlin under the control of a tetracycline-inducible promoter in a merlin-deficient meningioma cell line.

- d. The expression of merlin will be assessed in individual stable clones and clones expressing high levels of merlin will be chosen (Months 11-14).

We are currently in the process of characterizing merlin expression in stable KT21MG1 clones, and this screen will be completed as proposed by month 14.

Task 2. Establish an in vitro model of merlin loss in human arachnoidal cells and evaluate the consequent changes in cell growth, motility and survival (Months 1-36).

- a. Chemically synthesized siRNA for merlin will be obtained and the efficacy of these siRNA to downregulate merlin will be tested using transient transfections (Months 1-6).

Our plan was to use the GeneSuppressor kit from Imgenex for stable suppression of merlin. However, after a comprehensive screen of the literature and discussions with other researchers that have used this technology, we decided to use the pSuper RNAi System from Oligoengine (9, 10). This system provides a mammalian expression retroviral vector that directs intracellular synthesis of siRNA-like transcripts. It utilizes the polymerase-III H1-RNA gene promoter. Thus, the RNA produced lacks a polyA tail and has a termination signal consisting of five thymidines in a row. A transcript that resembles the ends of synthetic siRNAs is generated and causes efficient and specific down-regulation of gene expression. Chemically synthesizing siRNA is expensive and we opted for the cheaper strategy of directly subcloning siRNA oligonucleotides into the pSUPER retroviral expression vector and testing these constructs in transient transfection assays. NF2 and control siRNA were designed using the algorithm and RNAi design tools provided at the Oligoengine website (Table 2). Three different NF2-specific siRNA sequences and one control siRNA sequence was selected and subcloned into the pSUPER.retro.neo vector following manufacturer's directions. The sequences of the different siRNA constructs were confirmed by direct sequencing. Transient transfections using the lipofectamine-plus transfection agents were performed and NF2 transcript levels were quantitated 48 h post-transfection (Figure 7). We typically get transfection efficiencies of 20 to 40% of meningioma and arachnoidal cells using these conditions. NF2siRNA2 and NF2siRNA3 gave two and three-fold reduction in the levels of NF2 respectively while NF2shRNA1 was ineffective in reducing transcript levels of NF2. Therefore, we chose NF2siRNA2 and NF2siRNA3 for stable suppression of NF2 in arachnoidal and meningioma cell lines

- b. Merlin-specific siRNA plasmid will be constructed and stable clones expressing merlin siRNA will be generated in immortalized arachnoidal cells (Months 7-11).
- c. The extent of downregulation of merlin will be evaluated in individual stable clones and the clone with the greatest level of knockdown of merlin will be chosen (Months 12-14).

NF2siRNA2 and NF2siRNA3 were transfected into SA2 and SF6717 using standard retroviral mediated gene transfer and stable cell populations were isolated by selection with 600 µg/ml G418. Parallel control stables with a scrambled non-specific control siRNA sequence were generated. Transcript levels were assessed in the stable cell populations using quantitative PCR (Figure 8). Transcript levels were reduced by 50-fold and 14-fold in SA2 and SF6717 respectively using NF2siRNA3 when compared to controlsiRNA transfected stable cell populations. There was a concomitant reduction in protein levels of merlin as assessed by western blots

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(Figure 9) and immunofluorescence (Figure 10). Thus, we have successfully suppressed expression of merlin in arachnoidal cells and in one meningioma cell line using RNA interference technology.

In summary, we have successfully completed all the proposed tasks for year 1 of this three-year proposal. The experiments planned for the first phase involved characterizing meningioma and arachnoidal cells and establishing meningioma-specific NF2 model systems. In the next 2 years, these systems will be used for *in vitro* and *in vivo* functional studies and for identifying downstream effectors of NF2.

KEY RESEARCH ACCOMPLISHMENTS

- 1) We have successfully immortalized three additional meningioma cell lines using telomerase in conjunction with the E6/E7 oncogenes. Thus, we currently have a total of nine available meningioma cell lines.
- 2) Eight meningioma cell lines were positive for merlin expression. One meningioma cell line, KT21MG1, was merlin-deficient.
- 3) We have identified a novel NF2 splice site mutation in KT21MG1 that results in the insertion of 79 bp of intronic sequence in the NF2 cDNA and codes for truncated merlin protein.
- 4) We have expressed merlin in the KT21MG1 cell line using the tetracycline-inducible system.
- 5) Three different siRNA constructs were tested for their ability to silence merlin expression in arachnoidal cells and in a meningioma cell line. NF2siRNA3 resulted in the greatest suppression of merlin.
- 6) Stable suppression of NF2 in arachnoidal cells and the meningioma cell line, SF6717, has been achieved using NF2siRNA3 and the pSUPER retroviral system.
- 7) We have generated three different NF2 model systems in one arachnoidal cell line and two meningioma cell lines and will use these systems to investigate the function of merlin.

REPORTABLE OUTCOMES

- 1) We have developed three meningioma cell lines designated SF6717, SF6747 and SF6561.
- 2) We have also generated three sets of paired cell lines where the only difference is the expression of merlin. These are KT21MG1-Vector and KT21MG1-Merlin; SA2-ControlsiRNA1 and SA2-NF2siRNA3; and SF6717-ControlsiRNA1 and SF6717-NF2siRNA3.
- 3) The post-doctoral fellow, Katharine Striendinger-Melo has used the results from this research to apply for an international fellowship from the American Association of University Women. This one-year fellowship is only open to non-US citizens.

CONCLUSION

The aim of this proposal is to develop meningioma-specific NF2 model systems and to utilize these systems to investigate the tumor suppressive functions of NF2 in meningiomas. The first phase of this proposal is focused on characterizing and generating the relevant model systems. We have generated three new meningioma cell lines. Meningioma cell lines are rare and the addition of three new cell lines to the repertoire of meningioma cell lines is a resource needed by meningioma and NF2 researchers and is important to enhance functional and therapeutic investigations of this understudied brain tumor. We have characterized the merlin status of all available meningioma and arachnoidal cells. It is essential to know the merlin status of our *in vitro* model systems before we can develop NF2 model systems. Most meningioma cell lines were merlin-positive while KT21MG1 was merlin-deficient. We have therefore expressed merlin in KT21MG1 using a tetracycline-inducible expression system and have silenced merlin expression in SF6717 and in arachnoidal cells. We have characterized transcript and protein expression in these engineered cell lines and are poised to initiate *in vitro*

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and *in vivo* functional studies using these model systems. These model systems can also be used in the future to test potential novel therapeutics against NF2.

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Table 1. Summary of Available Meningioma and Arachnoidal Cell Lines and their NF2 status

Cell Line	Grade	Immortalization Strategy	NF2 Status		
			RNA	Western	IFC
IOMM-Lee ^a	3	None	+	+	+
KT21MG1 ^a	3	None	-	-	-
F5 ^a	3	None	+	+	+
SF3061 ^b	3	hTERT	+	+	+
SF6717 ^b	2	E6/E7 and hTERT	+	+	+
SF6747 ^b	2	E6/E7 and hTERT	+	+	+
SF6561 ^b	2	E6/E7 and hTERT	+	+	+
SF4068 ^b	1	E6/E7 and hTERT	+	+	+
SF4433 ^b	1	E6/E7 and hTERT	+	+	+
SA1 ^c		E6/E7 and hTERT	+	+	+
SA2 ^c		E6/E7 and hTERT	+	+	+
CA1 ^c		E6/E7 and hTERT	+	+	+

^aMalignant meningioma cell lines obtained from other researchers. ^bMeningioma cell lines generated in our laboratory. ^cArachnoidal cells isolated and immortalized in our laboratory. SA1 and SA2 were derived from surgically removed spinal arachnoid while CA1 was derived from surgically removed cranial arachnoid.

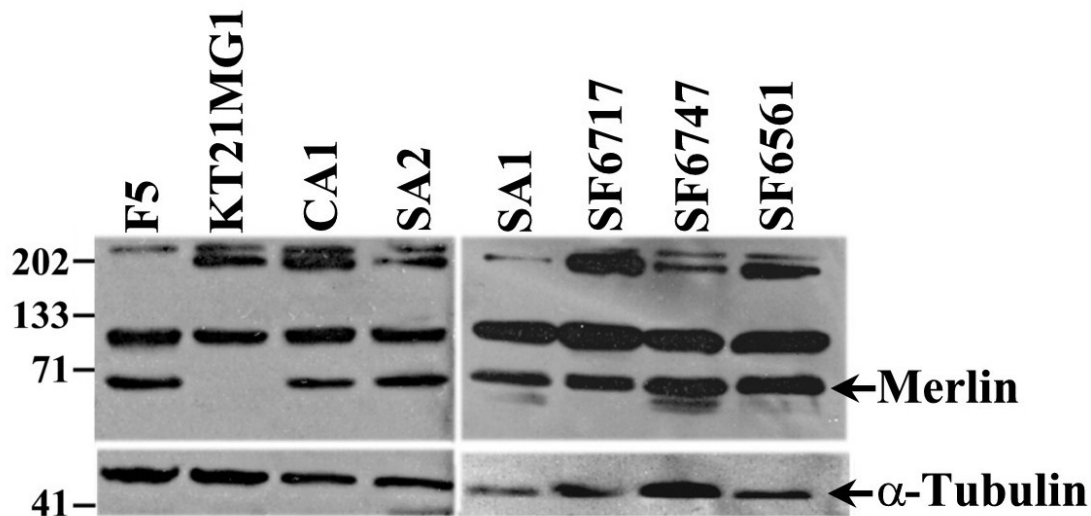


Figure 1. Protein expression of merlin in meningioma and arachnoidal cell lines. Protein levels of merlin isoform 1 in the meningioma cell lines F5, KT21MG1, SF6717, SF6747, SF6561 and in the arachnoidal cell lines SA1, SA2 and CA2 were measured by western blot analysis on total cell lysates using the A19 polyclonal antibody. α -tubulin was used as a control. The 68 KDa merlin band is labeled. Only KT21MG1 lacked expression of merlin.

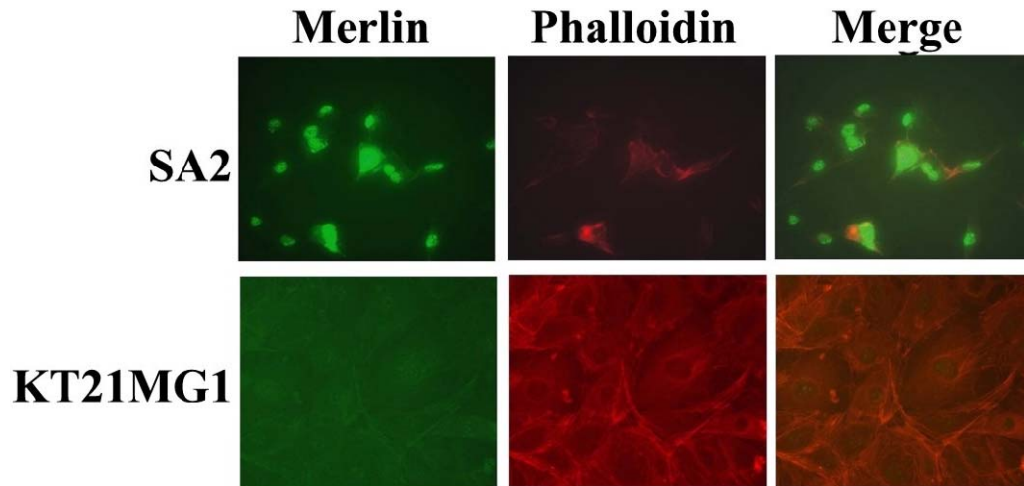


Figure 2. Protein expression as assessed by immunofluorescence in arachnoidal SA2 and meningioma KT21MG1 cells. Immunolabeling with the A19 antibody shows expression of merlin (green). Cells were costained for phalloidin (red). The merged image of merlin and phalloidin shows colocalization of merlin with F-actin at membrane ruffles in SA2 cells. In contrast KT21MG1 lacks expression of merlin.

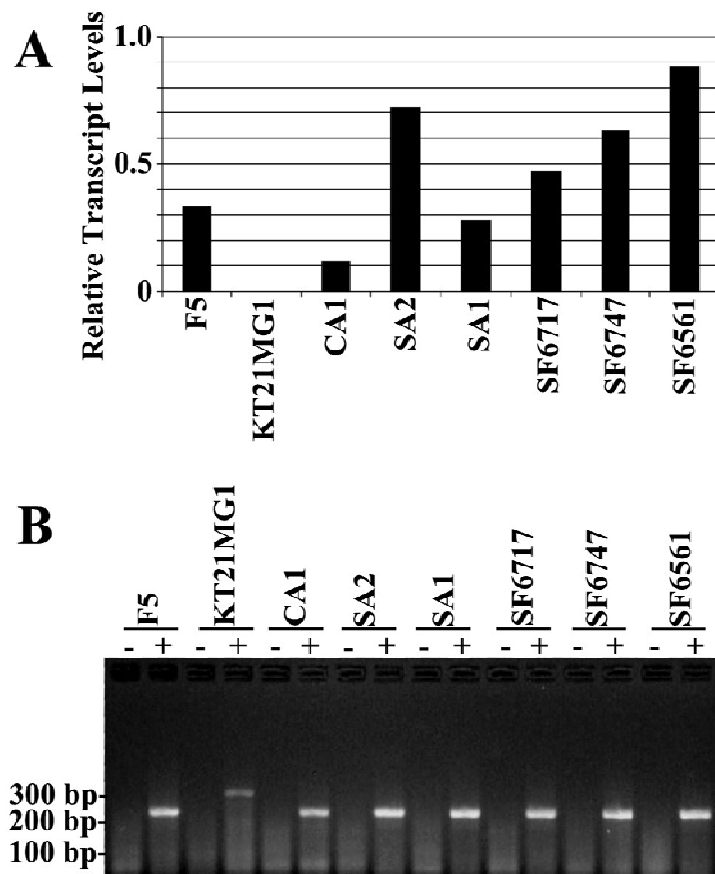


Figure 3. Transcript Levels of NF2 in meningioma cell lines.

A) Relative transcript levels of NF2 in meningioma cell lines (F5, KT21MG1, SF6717, SF6747, SF6561) and in arachnoidal cell lines (CA1, SA2, SA1) were compared using quantitative PCR. B) The corresponding NF2 PCR amplicons after 50 PCR cycles were resolved on an agarose gel. Note that the amplicon of KT21MG1 is larger than the expected 234 bp of NF2 amplicons (present in all the other cell lines) suggesting that NF2 has an insertion mutation.

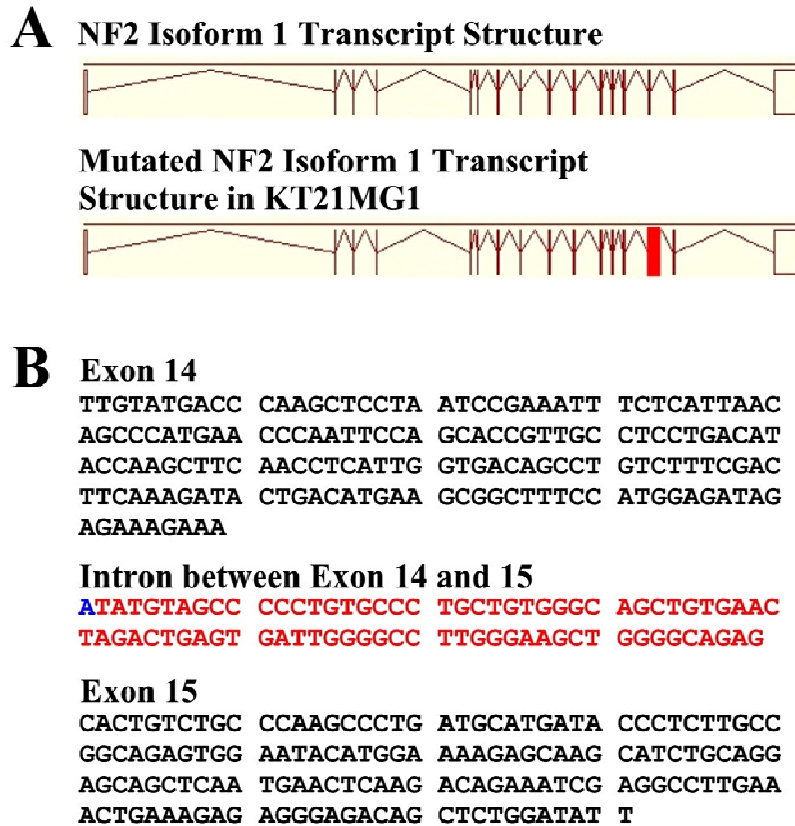


Figure 4. KT21MG1 transcripts revealed a splice mutation and partial intron retention. The sequence of the PCR amplicon of KT21MG1 shows a 79 bp insertion that matches the sequence of the initial part of the intron between exon 14 and exon 15 (in red). There is a substitution of the first G of the intron with an A (labeled in blue), which probably leads to cryptic splice utilization and partial intron retention reflected in the 79 bp insertion.

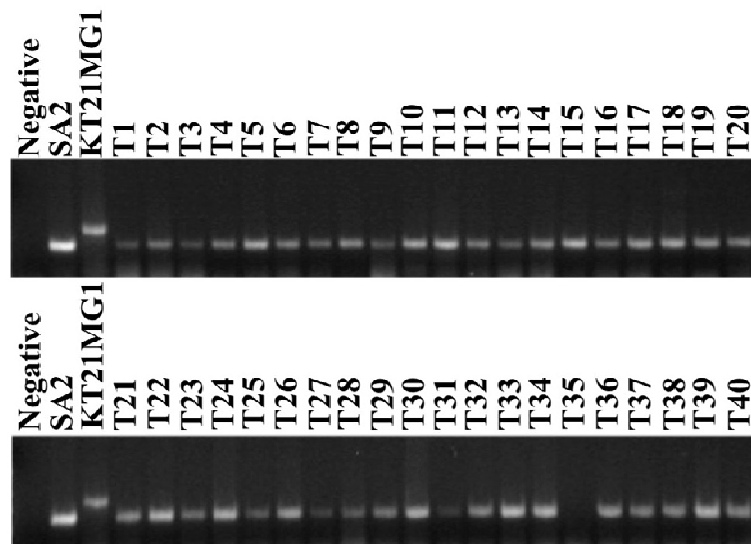


Figure 5. Insertion mutations between exon14 and 15 were not found in 40 primary meningioma sporadic tumors. Agarose gel showing PCR amplicons of 40 primary tumors (T1-T40) using primers that hybridize on exon 14 and exon 15. Control SA2 has the expected 234 bp product, while KT21MG1 has the mutated 313 bp product. None of the primary tumors show the insertion mutation found in the KT21MG1 cell line suggesting that this mutation is rare in meningiomas.

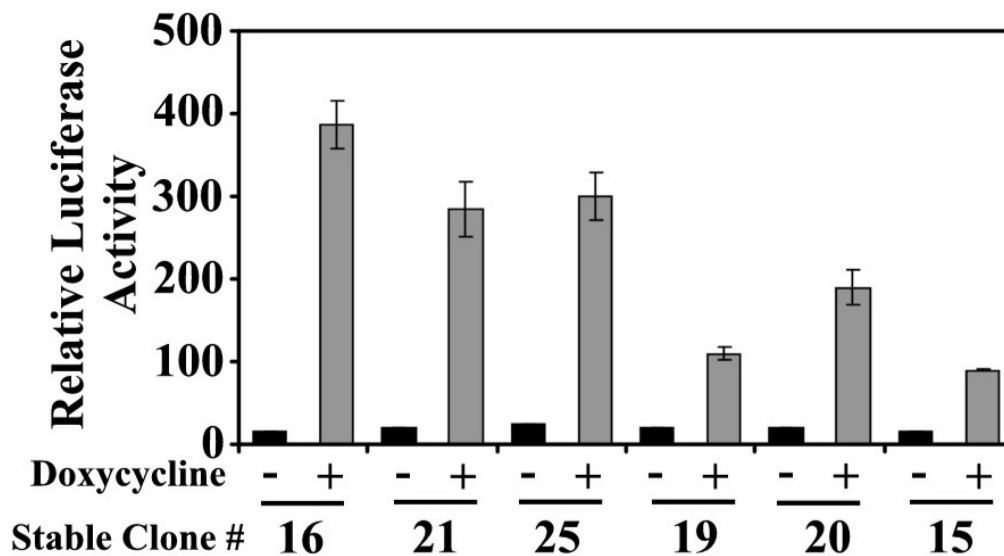


Figure 6. Fold induction of luciferase activity in different stable clones of KT21MG1 expressing the reverse tetracycline-transactivator (rtTA). Luciferase activity using a firefly-luciferase reporter was measured before (black bars) and after (grey bars) addition of Doxycycline (1 μ g/ml) in different stable clones of KT21MG1 transfected with rtTA. Expression levels were induced by 113 to 1163-fold in the different clones. A subset of the clones is shown here. Clone 16 has the highest luciferase activity after induction.

Table 2. Summary of NF2 and Control shRNA Constructs

Construct Name	Sequence of shRNA constructs	Match to NF2 Position (NM_000268)
NF2siRNA1	CTGCTGCAGATGAAAGAAG	1501-1519
NF2siRNA2	GCAGCAAGCACAATACCAT	2135-2153
NF2siRNA3	AGCAGCAAGCACAATACCA	2134-2152
ControlsiRNA1	GCGCGCTTTGTAGGATTCG	No Match

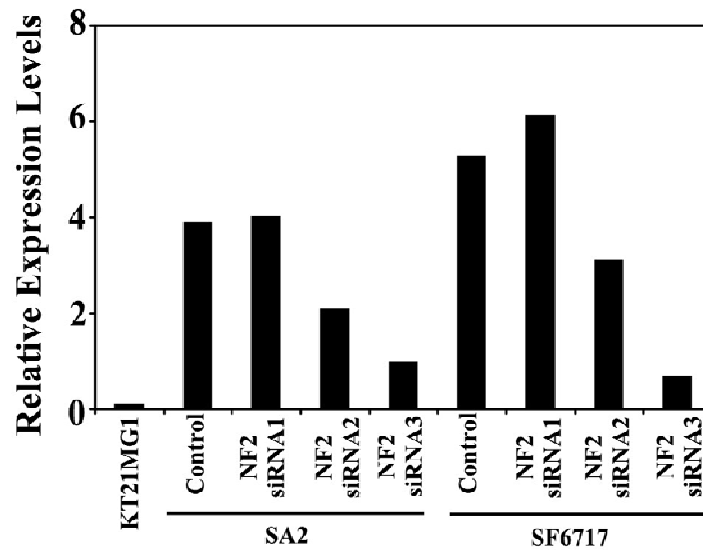


Figure 7. Transient expression of siRNA shows a decrease in NF2 transcript levels. Arachnoidal SA2 and meningioma SF6717 cell lines were transfected with three different NF2-specific siRNA sequences. The transcript levels of NF2 were evaluated by quantitative RT-PCR 48 h after transfection. NF2siRNA2 and NF2siRNA3 were effective in suppressing NF2 transcript levels compared to controls, while NF2siRNA1 did not achieve suppression of NF2.

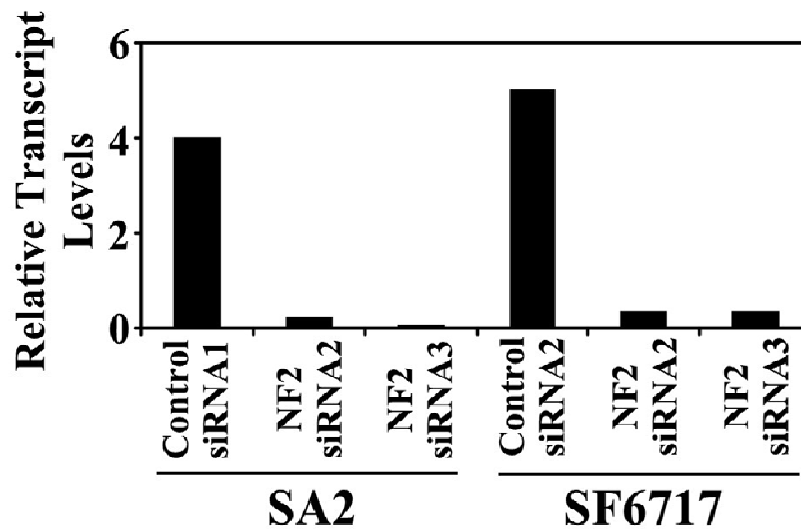


Figure 8. Stable suppression of NF2 after RNA interference. Stable cell populations expressing NF2siRNA2, NF2siRNA3 and ControlsiRNA1 were generated by retroviral mediated gene transfer followed by antibiotic selection. Transcript levels were reduced by 50-fold and 14-fold in SA2-NF2siRNA3 and SF6717-NF2siRNA3 respectively compared to controlsiRNA1 stables.

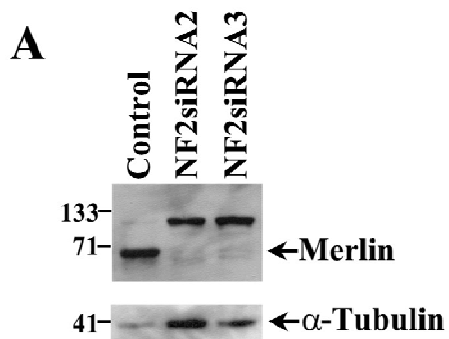


Figure 9. Stable suppression of Merlin by RNA interference. Merlin protein levels as assessed by Western blots are suppressed in SA2 (A) and SF6717 (B) cell lines after stable expression of the NF2siRNA2 and NF2siRNA3.

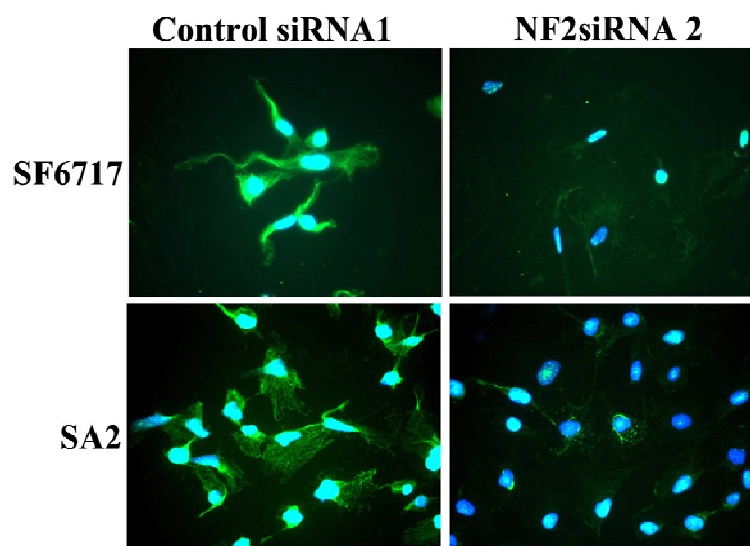
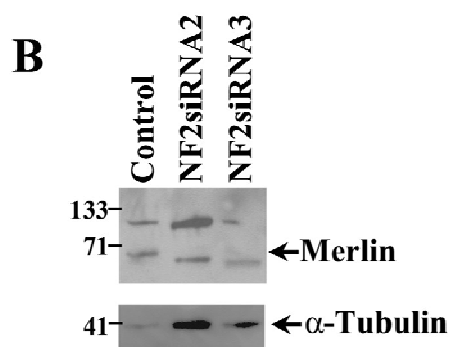


Figure 10. Specific suppression of merlin by RNA interference. Immunofluorescence with the A19 antibody shows merlin expression (green). The cells were counterstained with DAPI (blue). Note the almost complete absence of merlin in SA2-NF2siRNA2 and SF6717-NF2siRNA2 stable cell populations compared to controlsiRNA1 expressing cells.

Laboratory Investigation

A genetic strategy to overcome the senescence of primary meningioma cell cultures

Gilson S. Baia¹, Alison L. Slocum¹, Jeanette D. Hyer¹, Anjan Misra¹, Nouzhan Sehati¹, Scott R. Vandenberg^{1,2}, Burt G. Feuerstein^{1,3}, Dennis F. Deen¹, Michael W. McDermott¹ and Anita Lal¹

¹Brain Tumor Research Center, Department of Neurological Surgery, University of California, San Francisco, CA, 94143, USA; ²Department of Pathology, University of California, San Francisco, CA, 94143, USA; ³Department of Laboratory Medicine, University of California, 94143, San Francisco, CA, USA

Key words: immortalization, meningioma, model systems, senescence, telomerase

Summary

Even though meningiomas are the second most common brain tumor in adults, little is known about the molecular basis of their growth and development. The lack of suitable cell culture model systems is an impediment to this understanding. Most studies on meningiomas rely on primary, early passage cell lines that eventually senesce or a few established cell lines that have been derived from aggressive variants of meningiomas. We have isolated three primary meningioma cell lines that are negative for telomerase activity. We can overcome the senescence of a Grade III derived meningioma cell line by expressing the telomerase catalytic subunit (hTERT), whereas Grade I meningioma cell lines require the expression of the human papillomavirus E6 and E7 oncogenes in conjunction with hTERT. Meningioma cell lines, immortalized in this manner, maintain their pre-transfection morphology and form colonies *in vitro*. We have confirmed the meningotheial origin of these cell lines by assessing expression of vimentin and desmoplakin, characteristic markers for meningiomas. Additionally, we have karyotyped these cell lines using array CGH and shown that they represent a spectrum of the genetic diversity seen in primary meningiomas. Thus, these cell lines represent novel cellular reagents for investigating the molecular oncogenesis of meningiomas.

Introduction

Tumor model systems are essential to understand the function of genetic alterations in cancer, to assess their contribution to cancer progression and to serve as pre-clinical models for evaluating the toxicity and efficacy of conventional and novel therapeutic agents. Cell culture systems are convenient, reproducible and reliable, and they provide an important tool for initial investigations into the cancer of their origin. Meningiomas are the second most common adult brain tumor and are a considerable cause of morbidity and mortality, yet experimental investigations into their biology have been hampered by the lack of appropriate model systems [1]. Most studies on meningiomas have relied on primary, early passage cell lines that usually undergo cellular senescence after a few passages [2–5]. Thus, studies performed using these primary cell lines are not reproducible and characterizations of the cell lines are usually minimal. A few established meningioma cell lines are available and are commonly used by investigators in probing the biology of meningiomas [6, 7]. These cell lines have been derived from aggressive variants of meningiomas. For example, the commonly used meningioma line, IOMM-Lee, was derived from an intraosseous malignant meningioma [6]. While these cell lines are an invaluable resource, there is a need to develop additional cell lines that span the entire spectrum of meningiomas.

The purpose of this study was to overcome the senescence of primary meningioma cell lines and to develop meningioma cell culture model systems that are more characteristic of the majority of clinical meningiomas. Multiple pathways trigger cellular senescence and disruption of these pathways are required for immortalization [8]. Progressive telomere shortening is a primary cause of a finite proliferative life span and a telomere maintenance mechanism has been found in every immortalized cell line examined to date [9]. In the majority of immortalized cell lines and in ~85% of cancers, the enzyme telomerase is activated. In other cancers, telomere length is maintained by an alternative mechanism for lengthening of telomeres (ALT) [10]. Cell cycle checkpoint pathways also control cellular senescence, and cellular immortalization is commonly associated with disruption of the p53 and/or pRb pathways [11]. In fact, intact p53 and pRb pathways can trigger senescence in cells that have a telomere maintenance mechanism [12].

Here, we report that immortalization of a malignant meningioma cell line required expression of hTERT, while immortalization of two benign meningioma cell lines required disruption of the p53 and pRb pathways in addition to expression of hTERT. These immortalized cell lines maintain morphological, immunocytochemical and genetic features characteristic of meningiomas and represent a useful tool for gaining insights into the biology of this type of cancer.

Materials and methods

Cell line generation

Cell lines were established from fresh, viable tumor specimens that were confirmed by routine histopathologic evaluation to be meningiomas, using the 2000 WHO classification and grading system. Viable tissue was placed into Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (DME–10%FBS) within 90 s following surgical resection, transported to the lab and dissected into 1 mm pieces. Following digestion with DNase, Pronase and Collagenase for 30 min at 37 °C, the tissue fragment solution was filtered through a 100-micron filter, and the filtrate centrifuged at 1000 rpm for 10 min to collect dissociated cells. These cells were seeded at a density of 3 to 5×10^6 cells per 100 mm dish and maintained in DME–10%FBS. After achieving 80–90% confluence, the cultures were passaged 1:4. Each passage was considered as two population doublings.

Retroviral infections

Telomerase and E6/E7 expressing stable cell lines were generated using retroviral-mediated gene transfer. pWZL-blast-hTERT or pLXSP-puro-E6/E7 was transfected into the Phoenix A packaging cell line using lipofectamine. The 48 h culture supernatant was used to infect meningioma cell lines in the presence of polybrene (8 μ g/ml). Stable cell populations were selected using blasticidin (2 μ g/ml) for telomerase expression or puromycin (0.5 μ g/ml) for E6/E7 expression.

Telomerase activity

Telomerase activity was detected using the Telomeric Repeat Amplification Protocol (TRAP) assay. TRAP assays were performed using the TRAPeze kit (Intergen, Gaithersburg, MD) as described by the manufacturers. PCR products were resolved on a 10% polyacrylamide gel and viewed with SYBR Green I (Molecular Probes, Eugene, OR) staining.

Colony forming efficiency (CFE) assay

The CFE assay was used as described earlier with minor modifications [13]. Heavily X-irradiated (50 Gy) IOMM-Lee cells (3.5×10^4) were used as feeder cells and were plated 24 h in advance of seeding the experimental cells. The plates were incubated for 2 weeks before the colonies were stained with methylene blue. Colonies containing at least 50 cells were counted. Plating efficiency (PE) was calculated as the ratio of the number of colonies formed to the number of cells seeded.

Quantitative PCR

Quantitative PCR was performed on cDNA templates with the I-cycler machine (Bio-Rad, Hercules, CA) and SYBR Green I (Molecular Probes, Eugene, OR) using PCR conditions and data analysis as described earlier

[14]. The primers used for NF2 were 5'-ACCGTTGCC TCCTGACATAC and 5'-TCGGAGTTCTCATTG TGCAG and for hTERT were 5'-GGAAGAGTGT CTGGAGCAAG and 5'-GGATGAAGCGGAGTCTG GAC.

Immunofluorescence

Immunofluorescence was performed on meningioma cell lines for Vimentin using the clone Vim 3B4 antibody (0.5 μ g/ml, Dako Corporation, Carpinteria, CA), for Desmoplakin 1 and 2 using the clone DP1-1&2-2.15 antibody (20 μ g/ml, Research Diagnostics, Inc., Flanders, NJ) and for NF2 using the rabbit polyclonal NF2 (C-18) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Meningioma cell lines were grown in eight well chamber slides, fixed with 2% formaldehyde in Hanks balanced salt solution for 15 min at 37°C and permeabilized and blocked by incubation with 0.1% saponin, 10% FBS in PBS (Buffer A) for 15 min. The cells were then sequentially incubated with primary antibody and secondary antibody (4 μ g/ml; Alexa Fluor 594 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG), mounted in DAPI mounting media, examined and photographed with a Zeiss microscope.

Array CGH analysis

Genomic DNA was isolated from meningioma primary tumors or cell lines using the DNAeasy kit (Qiagen, Valencia, CA) following manufacturer's directions. Arrays spotted with 2464 mapped bacterial artificial chromosomes (BAC) covering the whole genome were hybridized with fluorescent labeled DNA, using 0.6 μ g of cell line DNA and 0.8 μ g of reference DNA from normal tissue [15]. The labeling reactions were performed overnight at 37 °C using Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, Piscataway, NJ) and the BioPrime kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and unincorporated nucleotides were removed using a Sephadex G50 column (Amersham Biosciences, Piscataway, NJ). Test and reference DNAs were mixed with 100 μ g of human Cot-1 DNA, ethanol precipitated, resuspended in 50 μ l of hybridization solution and applied to a pre-hybridized array. Arrays were incubated at 37 °C on a slowly rocking table for approximately 72 h. Arrays were washed in 50% formamide buffer. The spotted BACs were counterstained with 4',6'-diamino-2-phenylindole hydrochloride (DAPI). Finally, arrays were scanned and images processed by SPOT/SPROC custom software [16].

Results

Establishment of meningioma cell lines

Primary meningioma cell lines are readily cultured *in vitro*. These cell lines, however, have a limited life span usually senescing after several passages. In our laboratory, cell lines derived from Grade I meningiomas

typically senesce by population doubling 20, while cell lines derived from Grade III meningiomas senesce by population doubling 60 (data not shown). Most cancers have an activated telomere maintenance mechanism, often telomerase enzymatic activity, which is a marker of immortalization. We reasoned that meningioma cell lines undergo irreversible growth arrest because they are negative for telomerase activity and that reconstitution of this activity would immortalize meningioma cell cultures. We have derived three meningioma cell lines: SF3061-Parental, from a Grade III meningioma and SF4433-Parental and SF4068-Parental from Grade I meningiomas. All three cell lines were negative for telomerase activity, as assessed by the TRAP assay (Figure 1). IOMM-Lee, an established malignant meningioma cell line, is positive for telomerase activity.

We used retroviral-mediated gene transfer to engineer a derivative of SF3061-Parental, designated SF3061-hTERT, to express telomerase. SF3061-hTERT was positive for telomerase activity (Figure 2B). SF3061-Parental and SF3061-hTERT had an identical growth rate for 30 population doublings after which the parental cell line senesced while SF3061-hTERT con-

tinued growing with a steady population doubling time of 36 h (Figure 2C) for over 200 population doublings. Morphologically, SF3061-hTERT maintained the pattern and cytoarchitecture characteristic of the parental cell line (Figure 2A, a and b). Importantly, SF3061-hTERT formed colonies while the parental cell line did not form colonies (Figure 2D).

Next, we attempted immortalizing a Grade I meningioma derived cell line, SF4433-Parental, with telomerase. Telomerase-expressing SF4433 behaved like the parental cell line and did not grow beyond population doubling 20, suggesting that additional genetic changes are required for Grade I meningiomas to grow in culture. The human papillomavirus oncogene E6/E7 works by disrupting the retinoblastoma and the p53 pathways [17], both of which have been reported as being disrupted in malignant meningiomas [18]. We, therefore, transfected in the E6/E7 oncogenes in addition to telomerase into SF4433-Parental. The E6/E7 and telomerase expressing cell line (SF4433-E6/E7-hTERT) continued to grow in culture after the SF4433-Parental and SF4433-E6/E7 cell lines had senesced (Figure 3B). Once again, immortalized SF4433 maintained the morphology of the parental cell line (Figure 3A). SF4433-E6/E7-hTERT cells formed colonies (Figure 3C), whereas the parental cell line did not. Similar results

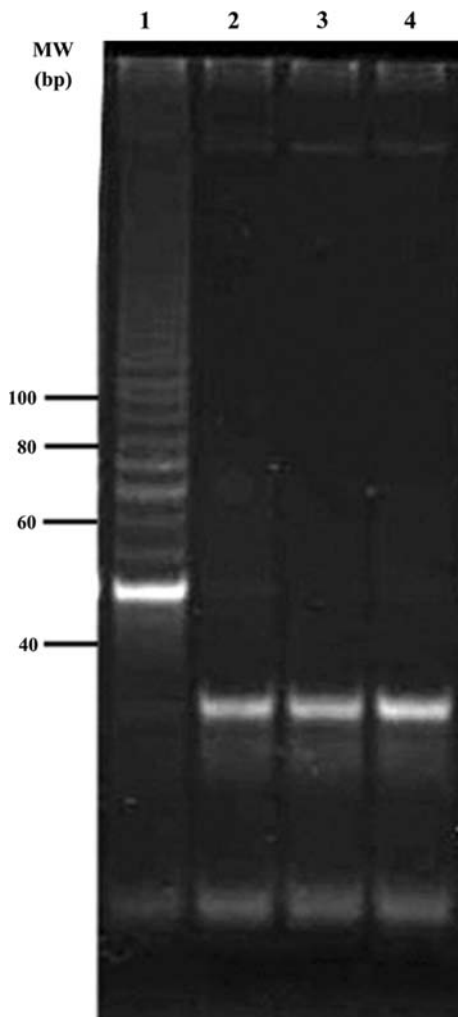


Figure 1. Meningioma cell lines, SF3061-Parental (2), SF4433-Parental (3) and SF4068-Parental (4) are negative for telomerase activity as assessed by the TRAP assay. IOMM-Lee (1), an established meningioma cell line, is telomerase positive.

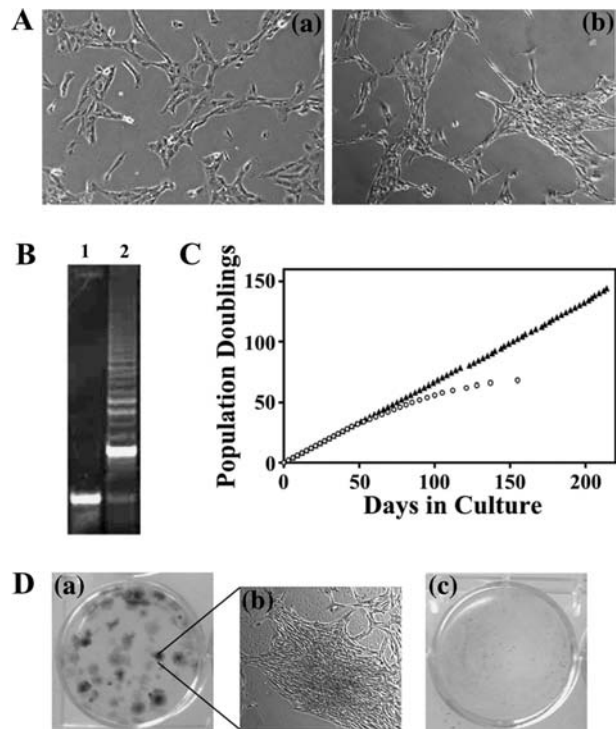


Figure 2. Expression of telomerase immortalizes SF3061-Parental, a malignant meningioma cell line. SF3061-Parental was established from a Grade III meningioma tumor and SF3061-hTERT was derived by stably expressing telomerase in SF3061-Parental. (A) The morphology of SF3061-Parental (a) is compared to SF3061-hTERT (b) at population doubling 22. Magnification is 100 $\times$. (B) Telomerase activity was measured using the TRAP assay for SF3061-Parental (1) and SF3061-hTERT (2). (C) Growth curve of SF3061-Parental (empty circles) and SF3061-hTERT (filled triangles). (D) CFE assay of SF3061-hTERT (a, b) and SF3061-Parental (c).

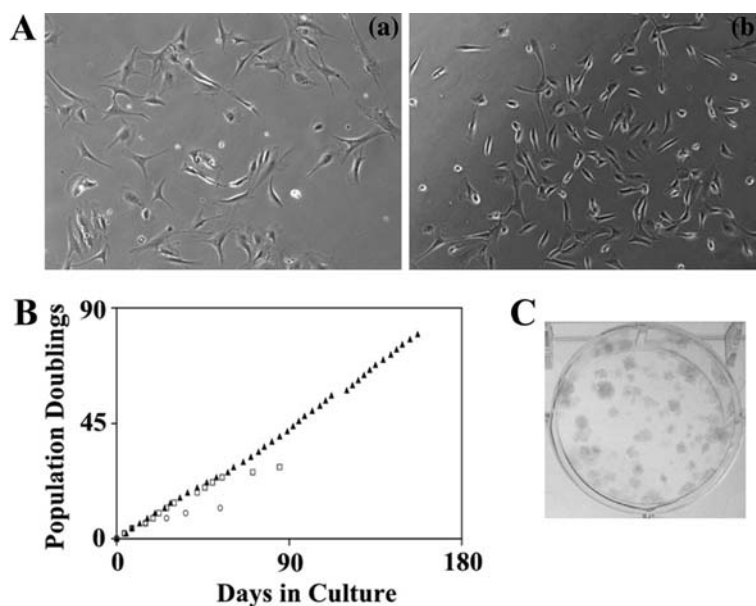


Figure 3. Immortalization of SF4433, a Grade I meningioma cell line. SF4433-Parental was established from a Grade I meningioma, SF4433-E6/E7 was derived from SF4433-Parental by stably transfecting the human papillomavirus E6/E7 oncogenes and SF4433-E6/E7-hTERT was derived by expressing telomerase in SF4433-E6/E7. (A) Morphology of SF4433-Parental (a) and SF4433-E6/E7-hTERT (b). Magnification is 100 $\times$. (B) Growth curve of SF4433-Parental (open circles), SF4433-E6/E7 (empty squares) and SF4433-E6/E7-hTERT (filled triangles). (C) CFE assay of SF4433-E6/E7-hTERT.

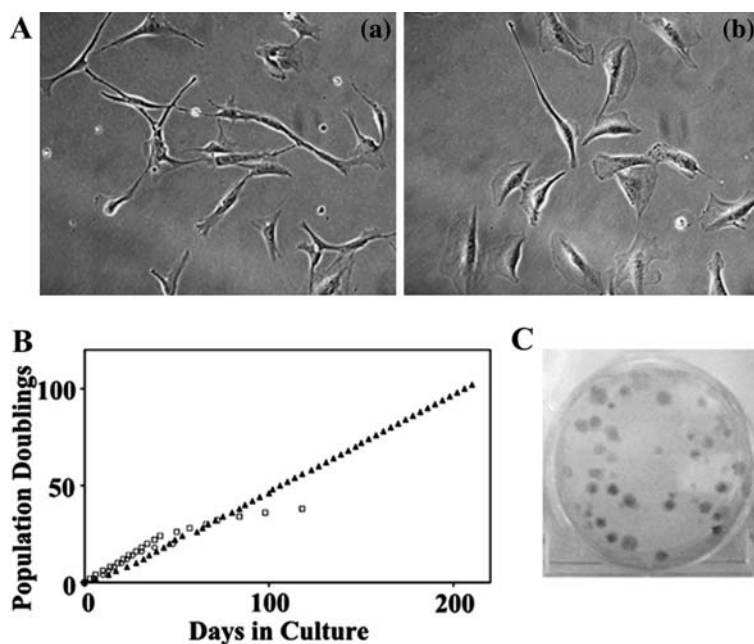


Figure 4. Immortalization of SF4068, a Grade I meningioma cell line. SF4068-Parental was established from a Grade I meningioma, SF4068-E6/E7 was derived from SF4068-Parental by stably transfecting the human papillomavirus E6/E7 oncogenes and SF4068-E6/E7-hTERT was derived by expressing telomerase in SF4068-E6/E7. (A) Morphology of SF4068-Parental (a) and SF4068-E6/E7-hTERT (b). Magnification is 200 $\times$. (B) Growth curve of SF4068-Parental (open circles), SF4068-E6/E7 (empty squares) and SF4068-E6/E7-hTERT (filled triangles). (C) CFE assay of SF4068-E6/E7-hTERT.

were obtained with SF4068, another Grade I meningioma derived cell line (Figure 4).

Primary meningioma tumors are positive for telomerase transcripts

The primary mode of repression of telomerase in human cells is through silencing of the hTERT gene via transcriptional repression [19]. Thus, quantitative PCR

analysis of transcript levels is well suited to evaluate the activation status of telomerase in normal and meningioma tissues. We used this technique to assess telomerase transcript levels in the primary meningioma tumors from which the cell lines were derived. While normal meninges and brain were negative for telomerase transcripts and no PCR amplimers were observed even after 50 amplification cycles, both SF3061 and SF4433 primary tumors were positive for telomerase transcripts

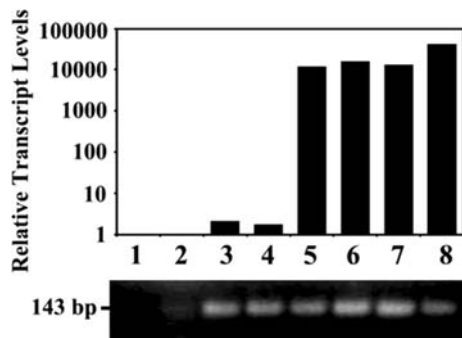


Figure 5. Telomerase expression in normal tissue, meningioma tumors and immortalized meningioma cell lines. Relative transcript levels of hTERT in non-neoplastic meninges (1), non-neoplastic brain (2), SF3061-Primary Tumor (3) SF4433-Primary Tumor (4), SF3061-hTERT (5), SF4433-E6/E7-hTERT (6), SF4068-E6/E7-hTERT (7) and IOMM-Lee (8) were compared using quantitative PCR. The corresponding 143 bp hTERT PCR amplicons after 50 PCR cycles were resolved on an agarose gel and are shown below.

(Figure 5). The telomerase transcript levels in these tumors were considerably lower than those in IOMM-Lee or in the hTERT-transduced meningioma cell lines (Figure 5).

Marker analysis of meningioma cell lines

Concomitant expression of vimentin, a class III intermediate filament protein and desmoplakin, a component

of desmosomal cell junctions, is a feature unique to arachnoidal cells and meningiomas [20–22]. Using immunofluorescence, we show that SF3061-hTERT, SF4433-E6/E7-hTERT and SF4068-E6/E7-hTERT express both vimentin and desmoplakin (Figure 6), confirming that the immortalized cell lines are meningotheelial in origin.

Karyotyping of meningioma cell lines

Array Comparative Genomic Hybridization (Array CGH) is a high-resolution genome-wide screening technique for the identification of amplifications and deletions of specific chromosomal regions [23, 24]. We have used this technique to karyotype the immortalized meningioma cell lines. The primary tumor from which the SF3061 cell line was derived had loss of entire chromosome 4 and 17, and losses of chromosome 9p24-p21, 11q23-qtel, 13q12-q21 (Figure 7). Interestingly, the SF3061-hTERT cell line had only a subset of the losses seen in the primary tumor (9p24-p21; 11q23-qtel; 13q12-q21; 17p). This confirmed that the cells that grew in culture bear a genomic correlation to the primary tumor. It also suggested that the SF3061 cell line was derived from a subpopulation of cells selected from the original heterogeneous tumor. We have karyotyped the commonly used IOMM-Lee cell line and compared the gross chromosomal abnormalities to that of SF3061 (Figure 7). While IOMM-Lee and the SF3061 primary tumor shared regions of loss on chromosome 4, this loss

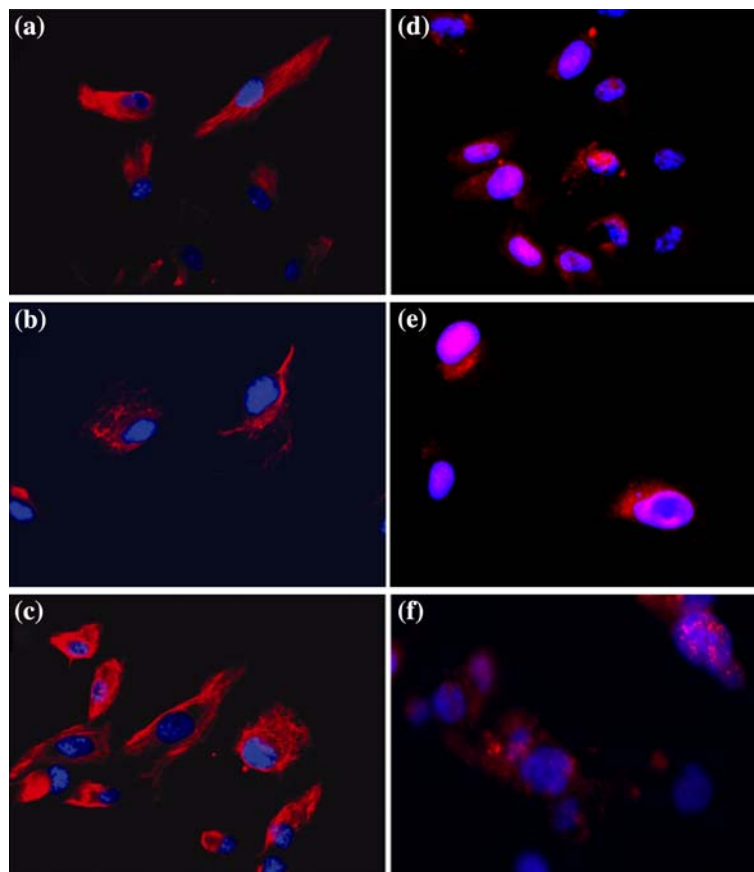


Figure 6. The meningeal origin of SF3061-hTERT (a, d), SF4433-E6/E7-hTERT (b, e) and SF4068-E6/E7-hTERT (c, f) was verified by staining for vimentin (a, b and c, red) and desmoplakin (d, e and f, red). Corresponding DAPI stains (blue) are overlaid.

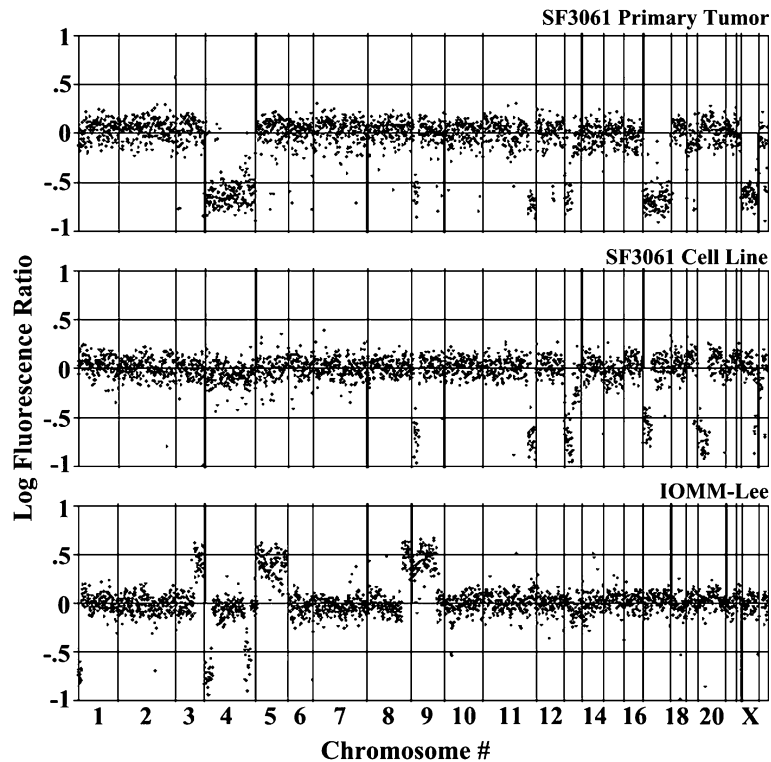


Figure 7. Karyotype of malignant meningioma cell lines. Array CGH analysis of genome copy number of SF3061 primary tumor, SF3061-hTERT cell line and the commonly used IOMM-Lee cell line are shown. The CGH ratio for each BAC array element is plotted as a function of its genome location, with chromosome 1 to the left and chromosome Y to the right, centromeric to telomeric. Vertical lines indicate chromosome boundaries.

was not conserved in the SF3061 cell line. The two cell lines had no chromosomal aberrations in common between them. IOMM-Lee had some characteristic higher-grade meningioma associated changes such as loss on 1p and gain on 9q, and SF3061-hTERT had the characteristic loss on 9p. Array CGH profiling of the Grade I primary meningioma from which SF4433 cell line was derived showed no gross chromosomal losses or

gains (Figure 8). This is a phenomenon often seen in benign meningiomas (unpublished data). We have karyotyped the SF4433 cell line and found that it also had no chromosomal abnormalities. SF4068, on the other hand, had gain of chromosome 5p and loss of chromosome 15 (Figure 8). None of our three cell lines had losses on chromosome 22, a region that is often deleted in meningiomas [25].

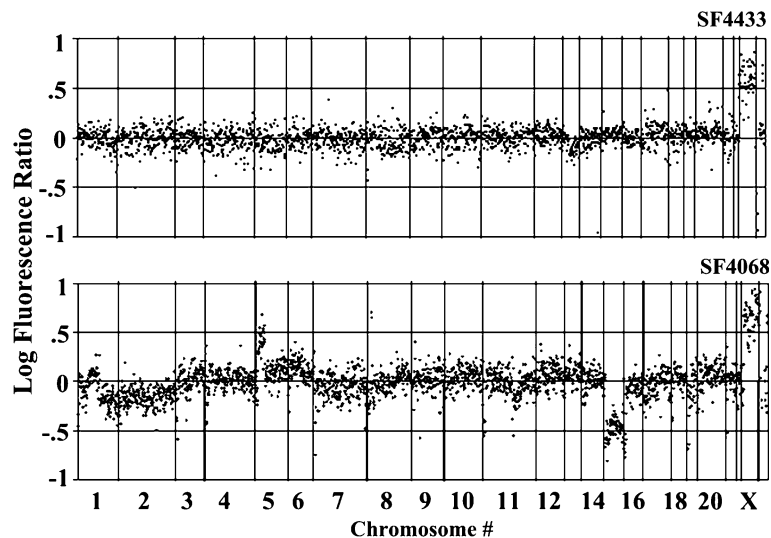


Figure 8. Karyotype of the immortalized benign meningioma cell lines. Array CGH analysis of genome copy number of SF4433 and SF4068 are shown. The CGH ratio for each BAC array element is plotted as a function of its genome location, with chromosome 1 to the left and chromosome Y to the right, centromeric to telomeric. Vertical lines indicate chromosome boundaries.

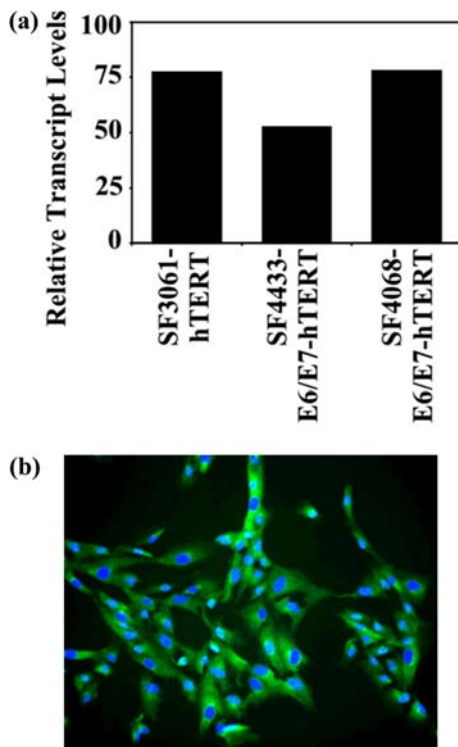


Figure 9. NF2 status of immortalized meningioma cell lines. (a) Relative transcript levels of NF2 were assessed in SF3061-hTERT, SF4433-E6/E7-hTERT, SF4068-E6/E7-hTERT using quantitative PCR. (b) Protein expression of NF2 (green) in SF3061-hTERT. Corresponding DAPI stain (blue) is overlaid.

NF2 status of meningioma cell lines

The neurofibromatosis 2 (NF2) gene located on chromosome 22 is frequently mutated in meningiomas [25]. Since we did not observe any deletions on chromosome 22 in our cell lines, we assessed the transcript and protein expression of NF2 in these cell lines. All three cell lines expressed the NF2 transcript, consistent with the observation that there were no deletions on chromosome 22 (Figure 9a). Using indirect immunofluorescence, all three cell lines were positive for the NF2 protein. A representative image of NF2 staining in SF3061-hTERT is shown in Figure 9b.

Discussion

Meningioma cell lines are notoriously resilient to growth in culture, undergoing irreversible growth arrest after a few passages *in vitro*. We have successfully produced three immortalized meningioma cell lines by inserting genetic elements that are commonly used to overcome cellular senescence and create tumor cells from normal cells [26]. Conversion of a normal human astrocyte to a human glioma requires the insertion of three key genetic elements: the expression of telomerase, functional inactivation of the p53/pRb pathways by the E6/E7 oncogenes and activation of the ras-signaling pathway by oncogenic H-ras [27]. In contrast, malignant and benign meningioma cell lines required insertion of one or two genetic changes, respectively. The other genetic chan-

ge(s) are presumably inherent to the meningioma tumor from which the cell line is derived. Thus, these cell culture model systems can be used to probe the biology of meningioma tumorigenesis and are a resource critically needed by the meningioma research community.

High levels of telomerase activity and expression have been related to histological grade and tumor recurrence in primary meningioma tumors [28]. Telomerase activity and hTERT mRNA have, however, been detected in some benign meningiomas [29]. In this study, the primary tumors from which our cell lines were derived had detectable transcript levels for telomerase, suggesting that at least a subpopulation of the original tumor was telomerase positive. However, the meningioma cells that survived and grew *in vitro* were telomerase negative. The tumor microenvironment is thought to play a significant role in the activation of telomerase. hTERT expression has been reported to be regulated by hormones, growth factors, differentiation-inducing agents and other tissue-specific environmental factors [30]. It is probable that hTERT expression and activity is driven by one or more of these factors in primary meningioma tumors, and that meningioma cells, once removed from their *in vivo* environment lose expression of telomerase. It is hypothesized that primary meningioma tumors contain immortalized cells but the difficulty in obtaining cell lines is a reflection of the lack of suitable cell culture conditions.

Established cell lines from benign meningiomas without genetic manipulation do not exist. There is only one recent study that reported the successful immortalization of a benign meningioma cell line with telomerase alone [31]. This is in contrast to our results where telomerase was insufficient to immortalize two independent benign meningioma cell lines and required additional genetic changes. This is probably a reflection of particular differences in the original tumors from which these cell lines were derived. While the cell line reported in the previous study had a deletion of chromosome 22, one of our cell lines had no gross chromosomal aberrations and the other had aberrations in regions other than chromosome 22.

Overcoming the cellular senescence of benign meningioma cell lines required disruption of the p53 and pRb pathways. Both these pathways are commonly disrupted in malignant meningiomas but not in benign meningiomas. The cyclin-dependent kinase inhibitors, CDKN2A (p16) and CDKN2B (p15), regulate cell cycle progression through the G1/S-phase checkpoint, and are lost in 46–56% of Grade III meningiomas [18, 32]. This checkpoint is also under the control of phosphorylation by the retinoblastoma protein (pRb). In the p53 pathway, p14^{ARF} is commonly deleted and/or mutated in malignant meningiomas [18, 32]. These genes are located on chromosome 9p, a region that is lost in the SF3061-primary tumor and cell line and consequently, SF3061 did not require disruption of these pathways for continued growth. An interesting question that arises from this study and previous observations is: What are the molecular mechanisms that allow benign meningiomas to evade cellular senescence and continue growing *in vivo*? Having access to benign meningioma cell lines

will allow meningioma researchers to begin answering this question.

The karyotypes of the three meningioma cell lines reported here are different from each other and from the established IOMM-Lee cell line. These cell lines reflect the genetic diversity seen in primary meningioma tumors. None of the cell lines reported in this study have deletions of chromosome 22 or loss of NF2, a frequent change in meningiomas. This is, however, not that surprising since approximately 40% of meningioma tumors do not have either aberrations of chromosome 22 or mutations in the NF2 gene [25, 33].

Insertion of genetic elements has undoubtedly altered some properties of the parental primary meningioma cell lines. Additionally, loss of p53 is known to enhance genomic instability [34] and it is likely that the immortalized cell lines will change characteristics over time in culture. Interpretation of results obtained using these cell lines should take these possibilities into consideration.

In conclusion, we report a genetic strategy that can be used to overcome the senescence of primary meningioma cell cultures. In addition to the cell lines reported in this study, it is anticipated that a repertoire of meningioma cell lines can be established and used to unravel the molecular basis of meningiomas.

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Address for offprints: Anita Lal, Brain Tumor Research Center, Department of Neurological Surgery, University of California, Box 0520 San Francisco, CA, 94143, USA; Tel.: +415-476-6662; Fax: +415-476-0388; E-mail: alal@itsa.ucsf.edu